AB21's Comments

Evaluation of the immunomodulatory effects of undecafluoro-2-methyl-3-oxahexanoic acid ("GenX") in C57BL/6 mice

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Abstract: Undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA[DJ1]), known as "GenX" by its U.S. manufacturer, is a compound designed to replace perfluorooctanoic acid (PFOA), a perfluoroalkyl compound that has been phased out of U.S. production due to environmental persistence, detectable serum concentrations in humans and wildlife, and reports of systemic toxicity. In experimental rodent models PFOA exposure suppresses T cell-dependent antibody responses (TDAR) and has been reported to suppress vaccine responses in exposed humans. To determine if U2M3-OHxA also modulates TDAR, male and female C57BL/6 mice were exposed

That's all?

Introduction

Per- and polyfluoroalkyl substances (PFASs) are anthropogenic organic compounds comprised of strong carbon-fluorine bonds that make them extremely useful as polymerization aids and surfactants for the processing of myriad consumer and industrial products. However, the characteristics that make PFASs beneficial in industrial processes make them problematic from an environmental health standpoint: perfluoroalkyl acids (PFAAs), a class of PFASs, are extremely persistent in the environment, and some bioaccumulate in wildlife and humans. They also have been associated with multisystem toxicity, most notably immunotoxicity, as similar effects on the immune system have been observed in both exposed experimental animal models and humans. Among the most well characterized PFASs are perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), which have been reported to potently suppress T cell-dependent antibody responses (TDAR) in experimental rodent models (DeWitt et al., 2008, 2009a, 2016; Loveless et al., 2008; Peden-Adams et al., 2008; Yang et al., 2002) and responses to vaccinations in exposed humans (Grandjean et al., 2012; Granum et al., 2013; Looker et al., 2014). Largely due to environmental persistence and growing reports of toxicity, PFOS was phased out of production by its major manufacturer in 2002 and PFOA was phased out of production by its major U.S. manufacturers in 2015. However, their usefulness as processing aids has prompted research into alternative PFASs with chemical characteristics that will reduce their toxicity.

The search for PFOA alternatives has focused on shorter-chain PFAAs and perfluoroether substances based on the assumption that they are less persistent and toxic (Stahl et al., 2009; Wang et al., 2015). However, evidence to date suggests that perfluoroether substituted chemicals may be just as persistent as PFAAs under environmentally relevant conditions (Wang et al.,

Laboratories (Raleigh, NC) at 7 weeks of age. Upon arrival to AAALAC accredited animal facilities at East Carolina University (ECU), mice were housed in groups of three and separated by sex. Distribution of animals from shipping containers was done semi-randomly, with the first animal being added to the first cage, the second to the second cage, the third to the third cage, and so forth. After distribution into cages, animals were weighed and redistributed to different cages to equalize body weights among cages so that pre-dosing body weights did not differ statistically by cage within sex ($p < 0.05$). Food and water were provided *ad libitum*, and animals were kept on a 12-hour light-dark cycle, 20-24°C, and a relative humidity of 60-65%. Bedding was changed twice weekly and the health of mice was monitored daily by both researchers and animal care staff. Mice were given five days to acclimate to their new housing arrangements before dosing began and the ECU Institutional Animal Care and Use Committee approved all procedures in advance.

Dosing: Mice were randomly assigned by cage to four different dose groups with six males and six females per group and two cages/sex/group housing three animals each. Doses were selected based on the reported NOAEL from the rat data extracted from the NPDES permit and reduced by an order of magnitude due to uncertainty about the sensitivity of mice relative to rats. Doses therefore were 0, 1, 10, or 100 mg/kg. U2M3-OHxA (CAS# 13252-13-6) was acquired from Synquest Laboratories (Alachua, FL, USA) and dosing solution was prepared fresh at the beginning of each week with sterile water and 0.5% Tween-20 vehicle to ensure emulsification into the dosing water. Concentrations of U2M3-OHxA dosing solutions were 0, 0.1, 1, and 10 mg/mL, which when given at 0.1 µL/g of body weight resulted in the appropriate mg/kg dose. Each mouse was dosed daily via oral gavage for 28 days. Body weights and deviations from

cytometer (BD Biosciences, San Jose, CA) and 10,000 events were collected from each sample. The total number of each cell type was determined from spleen cellularity.

IgM Antibody Response: On the 24th day after the initial dose, mice were immunized with sheep red blood cells (SRBCs) via tail vein injections. SRBCs were adjusted to 4 x 10^7 cells in 0.2 mL of sterile saline. Sera for measurement of SRBC-specific IgM antibodies was collected as described previously. IgM antibody titers were determined as described by DeWitt et al. (2016). Briefly, flat bottom 96-well Immunolon-2 ELISA microtiter plates (Dynatech Labs, Chantilly, VA) were coated with 125 µl of 2 µg/ml of SRBC membrane [1.46 mg/ml stock solution diluted in phosphate-buffered saline (PBS); prepared according to Temple et al. (1995)] and then incubated at 4°C for at least 16 hr. Each plate included 20 wells coated with pooled serum collected from healthy mice 5 d after primary immunization with SRBC, and 16 wells contained 100 µl PBS as blanks. After washing, blocking of non-specific binding, and addition of serum samples (serially diluted 1:2, starting at 1:8), secondary antibody (goat anti-mouse IgM horseradish peroxidase; Accurate Chemical and Scientific Corp., Westbury, NY) was added to the wells. Following three washes and addition of substrate [10 mg 2,2′-azino-di-(3 ethylbenz-thiazoline sulfonic acid, ABTS, Sigma) added to 50 ml phosphate-citrate buffer with one tablet of urea hydroxide peroxide (Sigma) in 100 ml distilled water, 0.05 M final solution], plates were incubated for 45 min at room temperature and then the absorbance in each well evaluated at 410 nm on a BioTek Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT). IgM anti-body titers were processed using SOFTmax Pro software (Molecular Devices, LLC, Sunnyvale, CA) to determine the $\log_2$ serum titers for an optical density of 0.5 U from the log-log curve of optical density versus dilution, as described by Temple et al. (1995).

animals/sex/dose). However, due to low blood collection volumes from individual animals at individual time points, serum concentrations reflect N = 1-6; concentrations based only on N = 1 are reflected in figure legends. Urine samples reflect pooled samples of three animals/cage and two pooled samples/dose as animals were housed three/cage with two cages/dose. Urine samples, therefore reflect N = 1 or 2 based on the volume of urine non-invasively excreted by animals on a particular collection day. Body weights over the course of the study were evaluated by two-way analysis of variance (ANOVA) with a repeated factor; all other data were evaluated separately within sex and across dose by one-way ANOVA with appropriate post-hoc t-tests when the overall ANOVA revealed an F-statistic with a p-value < 0.05.

Results

Body and Organ Endpoints

Body weight did not differ by dose within sex over the course of exposure or at experimental terminus (**Figure 2**), nor did animals show outward signs of systemic toxicity. Absolute (data not shown) and relative (**Figure 3**) liver weights were increased ($p < 0.0001$) by 40-160% in both males and females exposed to 10 or 100 mg/kg, with males having a greater percent increase relative to the 0 mg/kg group when compared to females. Absolute and relative thymus weights did not differ statistically by dose within either sex (data not shown). Absolute and relative spleen weights from females exposed to 100 mg/kg were 16.5% and 11.3% lower ($p < 0.002$) compared to weights from the 0 mg/kg group (**Table 1**); male spleen weights did not differ statistically. No statistical differences in splenic lymphocyte cellularity were noted across doses for either sex. Peroxisomal fatty acid oxidation also was increased in both males and females (**Figure 4**). Hepatic acyl-CoA oxidase activity was increased 122% and 222% in livers

mg/kg, maximal concentrations were reached after 5 days of exposure. In males, this was maintained until after 14 days of exposure whereas in females, serum concentrations, similarly to the 10 mg/kg group, dropped by about 50% from 5 to 14 days of exposure and by about 20% from 14 days of exposure to one day after exposure ended. In males, serum concentrations dropped by about 30% from 14 days of exposure to one day after exposure ended.

As full urine samples were not available on each day for all groups due to lack of urine excretion, it is challenging to meaningfully interpret urinary excretion trends. What is abundantly clear from the urine data, however, is that males excreted a much higher concentration of U2M3-OHxA across all concentrations and time points collected relative to females (**Figure 7**).

Discussion

U2M3-OHxA is a compound that was designed to replace PFOA in various manufacturing processes and is marketed as a processing aid with a toxicological profile more favorable than PFOA and that has rapid bioelimination. It was introduced to fluoropolymer manufacturing processes in 2009 and, to our knowledge, no peer-reviewed published data about its toxicological effects exist. In our study, we chose the C57Bl/6 mouse model as it is a rodent model that is sensitive to the immunomodulatory effects of PFOA exposure (DeWitt et al., 2009b). We also chose to evaluate the U2M3-OHxA compound following a standard immunotoxicity testing protocol, employing a 28-day exposure duration and evaluating antigen-specific antibody responses (i.e., TDAR) and enumeration of splenic lymphocytes. TDAR also was chosen as suppression of the TDAR has been reported in various rodent species after exposure to ammonium perfluorooctanoate (APFO, the ammonium salt of PFOA), PFOA, or PFOS (DeWitt et al., 2008, 2009a, 2016; Loveless et al., 2008; Peden-Adams et al., 2008; Yang et al., 2002a)

to either 0.94 or 1.88 mg/kg of PFOA via drinking water for 15 days increased absolute and relative liver weights in female C57Bl/6 mice when evaluated at the end of the study (DeWitt et al., 2008). A higher dose of PFOA, 40 mg/kg, was reported to increase liver weights in male C57Bl/6 mice after only two days of exposure (Yang et al., 2000) and liver weight increases were reported in male Crl:CD-1(ICR)BR given APFO for 29 days (Loveless et al., 2008). Hepatic acyl-CoA oxidase activity (**Figure 4**), a marker of hepatic peroxisome proliferation, was increased in both males and females exposed to 100 mg/kg and in males exposed to 10 mg/kg. [CH3]An earlier study on the potential immunotoxicity of PFOA reported increases in hepatic acyl-Co-A at a low dose of about 2 mg/kg after 10 days of dietary exposure in male C57Bl/6 mice (Yang et al., 2001). In terms of liver endpoints typically evaluated after PFOA exposure, it appears as if U2M3-OHxA is not as potent in inducing either hepatomegaly or hepatic peroxisome proliferation as PFOA.

Lymphoid organs also appeared to be less sensitive to U2M3-OHxA exposure than to PFOA exposure. The high dose (100 mg/kg) of U2M3-OHxA was associated with a reduction in absolute and relative spleen weights in female animals whereas PFOA and APFO have been reported to reduce both spleen and thymus weights at lower doses (10 – 40 mg/kg) and after shorter or similar exposure durations (DeWitt et al., 2008; Loveless et al., 2008; Yang et al., 2000, 2001). It is probable, given the weight loss associated with these relatively higher doses of PFOA, that lymphoid organ atrophy occurred as a result of systemic toxicity rather than immune system-specific effects of PFOA exposure.

The effect of PFOA exposure on antigen-specific antibody production has been documented in several strains of mice and is supported by epidemiological studies that associated serum PFOA concentrations with reduced responses to vaccines. These data are

was relatively mild; IgM production was 7.3% lower in treated animals relative to control animals. This suppression also was not associated with a reduction in body weight or overt signs of systemic toxicity. Relative to PFOA, U2M3-OHxA appears to be much less potent at affecting the TDAR.

Of particular interest regarding the effects of PFOA on the TDAR is that IgM antibody suppression occurs at doses that do not produce notable effects on splenic lymphocyte numbers as well as T and B cell subtypes. Higher doses of PFOA and APFO have been associated with reduced splenic and thymic lymphocyte numbers (Loveless et al., 2008; Yang et al., 2000, 2001) as well as reductions in T and B cell subtypes (Yang et al., 2001). However, lower doses of PFOA have not been reported to consistently alter lymphocyte numbers in lymphoid organs or T and B cell subtypes (DeWitt et al., 2016). In this study, U2M3-OHxA exposure failed to alter splenic lymphocyte numbers or T cell subpopulations when compared to control values. In male animals, however, the percentage of B cells was reduced by exposure to 1 or 100 mg/kg of U2M3-OHxA; animals exposed to 10 mg/kg had a small reduction in the percentage of B cells (2.5%) that was not statistically significant. The reductions in animals exposed to 1 and 100 mg/kg were mild, 5.7% and 10.1% respectively, and were not observed in female animals, even at the dose that was associated with a reduction in the TDAR. These data suggest that U2M3-OHxA may affect the TDAR via different pathways than does PFOA. Several studies have attempted to determine the mechanism(s) by which PFOA impacts the TDAR and to date, no consensus has been demonstrated by the data. Some studies (Yang et al., 2002b; DeWitt et al., 2016) have attempted to link peroxisome proliferation via activation of the peroxisome proliferator activated receptor alpha (PPARα) with PFOA-induced suppression of T and B cell subpopulations or the TDAR, but even though PPARα-null animals have somewhat attenuated

compared to PFOA. Our data support this general trend although our study was not conducted according to pharmacokinetic study guidelines. In our study, male animals had much higher serum and urine concentrations as the study progressed relative to female animals, suggesting slower plasma and therefore urinary clearance. Relative to PFOA, and in support of the Gannon et al. (2016) study, it appears as if U2M3-OHxA has a shorter serum half-life and more rapid excretion, at least in mice. It appears as if U2M3-OHxA is accumulated and excreted at a different rate in male and female mice; pharmacokinetic studies and serum measures of PFOA suggest that male and female mice accumulate and excrete PFOA fairly similarly. Lau et al. (2006) reported relatively similar serum concentrations in male and female CD1 mice given 20 mg/kg of PFOA for 17 days. Serum PFOA concentration in male mice was ~200 µg/mL and was ~175 µg/mL in female mice, a difference of about 12% (Lau et al., 2006). In our study serum U2M3-OHxA concentration in the 10 mg/kg group after 14 days of exposure (the closest comparator in dose and time) was about 65% greater in males than in females. Certainly this difference between PFOA and U2M3-OHxA could be strain-dependent or concentration-dependent. However, these data, combined with the results of the Gannon et al. (2016) study, indicate that unlike PFOA, male and female mice accumulate and excrete U2M3-OHxA at very different rates. In previous studies with experimental rodent models, rats were the only species with significant sex-related differences in urinary excretion of PFOA. This difference is thought to be testosterone-dependent at the level of the renal tubular cells as castrated male rats excrete PFOA more rapidly and when treated with testosterone, excrete PFOA more slowly (Kudo and Kawashima, 2003). The results of our study and the Gannon et al. (2016) study suggest that U2M3-OHxA also may induce sex-specific effects. Therefore, future studies with mice exposed to U2M3-OHxA should evaluate toxicological endpoints in both sexes.

necessarily reflect the views and policies of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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Table 1. Splenic lymphocyte subpopulations in adult C57Bl/6 male and female mice orally exposed to undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA) for 28 days.

U2M3-OHxA	Relative spleen wt.	Cells/spleen (10^6)	B Cells	CD4+/CD8-	CD4-/CD8+	CD4+/CD8+	CD4-/CD8-
Males							
0 mg/kg	3.81 (0.40)	38.03 (11.57)	66.78 (2.89)	57.17 (3.75)	34.79 (1.92)	1.48 (0.20)	6.57 (1.98)
1 mg/kg	3.59 (0.61)	46.66 (17.59)	62.97 (2.26)*	55.49 (3.55)	34.79 (2.69)	1.25 (0.16)	8.47 (1.41)
10 mg/kg	3.78 (0.47)	41.83 (14.01)	65.05 (2.21)	56.66 (1.68)	34.08 (1.43)	1.40 (0.44)	7.87 (0.74)
100 mg/kg	4.07 (0.99)	54.43 (17.88)	60.03 (2.08)*	56.30 (5.03)	33.68 (2.31)	1.61 (0.33)	8.41 (2.87)
Females							
0 mg/kg	5.34 (0.39)	45.18 (14.78)	67.22 (1.44)	52.69 (3.17)	32.45 (1.03)	1.19 (0.19)	13.67 (2.76)
1 mg/kg	5.15 (0.56)	58.83 (9.31)	68.49 (3.38)	54.16 (4.47)	31.68 (1.56)	1.35 (0.34)	12.47 (3.78)
10 mg/kg	5.01 (0.77)	51.16 (12.05)	66.987 (3.00)	51.89 (5.08)	32.53 (1.96)	1.45 (0.82)	12.96 (5.23)
100 mg/kg	4.65 (0.56)*	49.76 (12.41)	68.57 (1.80)	54.72 (3.86)	31.86 (1.86)	1.45 (0.50)	11.97 (3.35)

Data are presented as the mean percent gated cells (standard deviation) based on a total of 10,000 events collected. N = 12 animals/sex/dose.

*Statistically different ($p < 0.05$) from the 0 mg/kg group of the same sex.

Figure Legends

Figure 1. Chemical structure of undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA).

Figure 2. Body weights (mean ± SD) of male or female C57Bl/6 mice given undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA) via gavage for 28 days. No statistically significant differences in mean body weights collected at study terminus (one day after exposure ended) were associated with U2M3-OHxA exposure.

Figure 3. Relative liver weights (absolute liver weight adjusted for body weight; mean ± SD) of male or female C57Bl/6 mice given undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA) via gavage for 28 days. Livers were collected one day after exposure ended. * indicates a statistically significant increase in relative liver weight relative to the 0 mg/kg group of the matching sex ($p < 0.05$).

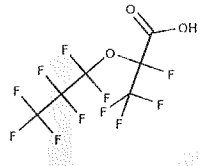
Figure 4. Hepatic peroxisome proliferation (mean ± SD) of male or female C57Bl/6 mice given undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA) via gavage for 28 days. Acyl-CoA oxidase activity was measured in archived livers that had been collected from animals one day after exposure ended. * indicates a statistically significant increase in acyl-CoA oxidase activity relative to the 0 mg/kg group of the matching sex ($p < 0.05$).

Figure 5. Antigen-specific IgM antibody production (mean ± SD) of male or female C57Bl/6 mice given undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA) via gavage for 28 days. IgM serum titers were measured in archived sera that had been collected from animals one day after exposure ended. * indicates a statistically significant reduction in IgM serum titer relative to the 0 mg/kg group of the matching sex ($p < 0.05$).

Figure 6. Serum concentrations (mean ± SD) of male or female C57Bl/6 mice given undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA) via gavage for 28 days. Sera samples were collected after 1, 5, 14, and 28 days of exposure. A) Males. B) Females * indicates a statistically significant increase in serum concentrations relative to the 0 mg/kg group at the same time point ($p < 0.05$). N = 1-6/dose. Samples where N = 1 occurred only in females on day 5 for the 10 and 100 mg/kg dose groups.

Figure 7. Urine concentrations (mean) of male or female C57Bl/6 mice given undecafluoro-2-methyl-3-oxahexanoic acid (U2M3-OHxA) via gavage for 28 days. Urine samples were collected after 1, 2, 3, 5, 10, and 14 days of exposure. A) Males. B) Females. Samples are pooled samples of three animals/sex/cage and two pooled samples/dose; sample sizes are therefore N = 1 or 2/dose depending on the quantity of urine non-invasively excreted on a particular sample collection day.

Figure 1



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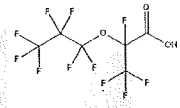

CPH version

Battle of the structures!

Which version should be the submitted version?

Figure 2

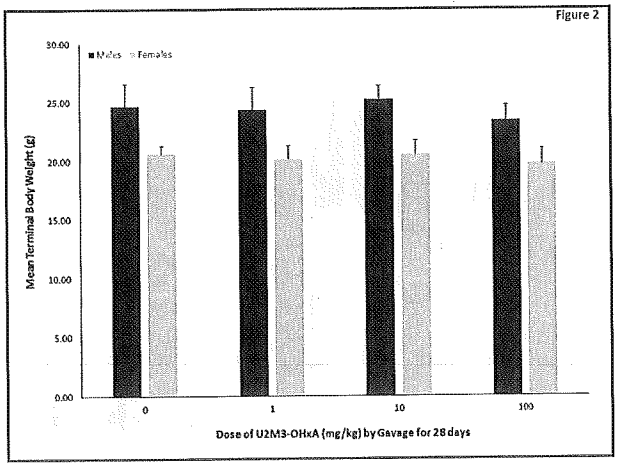

Figure 3